J A G I E L L O N I A N U N I V E R S I T Y
I n s t i t u t e o f C h e m i s t r y
Karasia 3 30-060 K r a k ó w, Poland

4501

Bronisław Zapiór, Adam Juszkiewicz, Jadwiga Potaczek

INVESTIGATION OF THE EFFECTS OF PROCESSING ON THE HYDRATION OF PROTEINS BY THE USE OF ULTRASONIC INTERFEROMETRY TO OBTAIN BASIC INFORMATION USEFUL FOR IMPROVED CONCENTRATED MILK PRODUCTS DEVELOPMENT

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F I N A L R E P O R T

The presented paper concerns physicochemical investigations of milk and milk proteins using the acoustic method and other investigative methods which were applied with the aim of verifying the conclusions drawn from the acoustic measurements. The investigation consists of four parts.

Part I. Model studies of protein hydration using the acoustic method. 1 - 23

In this part of the study the acoustic method of hydration measurement was described and the results of hydration number measurements for carboxylic acids, amino acids, amides, sugars, olygopeptides and simple macromolecules were given. The numbers of water molecules bound with the particular functional groups occurring in proteins were determined. The existence of hydration of alkyl groups was proved. Measurements in macromolecular solutions using acoustic and NMR methods showed that hydration was independent of the molecular weight of the polymers studied.

Part II. Investigation of hydration of casein and its fractions. 24-28

Hydration for isoelectric casein and α_s, β-, $\varkappa$ -casein fractions was determined. The method of preparation of particular casein fractions was described. In this group of investigations hydration measurements for solids-no-fat total milk components were also performed. The measurements did not show substantial differences of hydration number values for casein and its fractions.

Part III. Model investigations of detergent-casein interactions. 29-45

Measurements were carried out in casein-detergent system. Investigations were performed using three methods: acoustic, tensometric and equilibrium dialysis. Measurements showed that there exist interactions of a hydrophobic type between casein and different types of cationic, anionic and non-ionic detergents.

Part IV. Investigations of the souring process of not fat milk. 46-48

The souring process of milk was studied by use of ultrasound velocity and pH measurements. Investigations comprised ultrasound velocity and pH measurements of milk as a function of storage time, as well as measurements for milk to which acetic acid or lactic acid was added. The above was performed with the aim of checking whether the observed changes of ultrasound velocity during the souring process of milk are the result of pH changes which are caused by the influence of acids created during the souring process or else if they are the result of other deciding factors. Measurements indicated that the cause of ultrasound velocity changes near the precipitation point was the reorientation of casein micelles and order increase.

Part I. Model studies of protein hydration using the acoustic method.

Introduction

Ultrasound interferometry can be a useful instrument for investigations of hydration of substances in an aqueous environment. The advantage of this particular method of hydration number determination is its great simplicity. The first method of determining hydration number using ultrasound velocity and density measurements was proposed by Passynsky about fourty years ago [1,2]. Passynsky assumed that the decrease of solution compressibility is connected with the strong compression of water in an ionic field and that this decrease of compressibility in solution is due to hydration.
He found the following expression for determining the hydration numbers of molecules in aqueous solution:

$$n_h = \left(1 - \beta_T / \beta^0_T\right) \frac{100 - x}{M_0} \cdot \frac{M_2}{x} \qquad /1/$$

where β_T and β^0_T are the isothermal coefficients of compressibility of solution and solvent respectively, x is the weight percent of the solute, M_0 is the molecular weight of the solvent and M_2 is the molecular weight of the solute.
All of the remaining methods using ultrasound measurements for the determination of hydration numbers are based on this expresion [3 - 19]. In deriving this expression Passynsky assumed that an ion together with its hydration shell is incompressible. The other authors assumed ionic incompressibility but the compressibility of water in the hydration shell they accepted to be equal to the compressibility of free water. The incompressibility of an hydrated ion can only be valid for small ions such as: Mg^{2+} and Li^{+}. In the case of larger ions the electric field of these ions interacting with water molecules changes widely and the local compressibility of water molecules in the hydration shells also changes / 0 - 45 x $\times 10^{12}$ dyne^{-1}cm^2 / [20]. For the molecules of non-electrolytes and macromolecules the situation is still more complicated

because except for the necessity of taking into consideration the compressibility of water in the hydration shell, the compressibility of the solute has also to be considered. Generally it was believed that the small molecules of non--electrolytes are incompressible and the compressibility of hydration water is equal to the compressibility of normal ice [10,11]. Thus, the hydration numbers of electrolytes and non-electrolytes obtained by different authors on the basis of different assumptions can not be treated as real because the possibility of experimental verification of these assumptions does not exist. Also in the other methods of the determination of hydration numbers [5,6,21,22] e.g. from the measurements of partial molar volumes [23 - 30,33], viscosity [31,32] mobility [32,34,35], selfdiffusion coefficients [36], dielectric constants [37 - 41], n.m.r. [39, 42 - 50] and activity coefficients [51 - 55] varied assumptions are accepted which can not be verified experimentally and the hydration numbers obtained by these methods differ from each other sometimes markedly. None of these methods exept n.m.r. can be applied simultaneously to electrolytes, non-electrolytes and macromolecules. For these reasons the method of measurement of hydration numbers which was first proposed by Yasunaga et.al. [56] was worked out. This method gives believable results for electrolytes as well as for non-electrolytes and macromolecules. Using this method the hydration of molecules can be determined by ultrasound velocity measurements in alcohol-water solutions. It is known [57,58] that the dependence of ultrasound velocity as a function of alcohol concentration in water-alcohol mixtures is parabolic and the maximum is precisely defined for each temperature. The difference between the abscissae of the maxima of the curves obtained is caused by the molecules of the solute bonding part of the water.
With this assumption we have:

$$\frac{A_o}{W_o} = \frac{A_1}{W_1 - W_x} = \text{const}$$

where A_o and W_o are the amounts of alcohol and water corresponding to the maximum for alcohol-water mixtures without solute,

A_1 and W_1 are the amounts of alcohol and water at the maximum for alcohol-water solutions containing a certain amount of solute. W_x in this equation is the amount of water bound to solute.

The structure of alcohol-water solutions.

The addition of a small amount of a non-electrolyte to water causes stabilization of the structure of water [59 - 63]. By stabilization of the structure of water is understood effects connected with the passage of non-electrolyte molecules into the cages of this structure. According to Danford and Levy [63] the structure of water /particulary its short-range order/ ought to be considered as a defective ice structure with partly filled cages. In the structure of ice every cage is surrounded by six water molecules. Thus, every molecule neighbors with three cages and therefore the number of cages in the structure of ice is two times smaller than the number of molecules. In water some of the molecules form a skeleton of cages and some fill up the cages [64 - 67] . The presence of non-electrolyte molecules in the cages leads to the decrease of translational mobility of water molecules. In effect it causes the decrease of the selfdiffusion coefficient of water and changes of kinetic and equilibrium properties of solution. For alcohol solutions an anomalic course of some properties of solution can be observed within the concentration range of 0.03 to 0.2 mole fraction of alcohol. In Fig.1 the anomalic course of several chosen physicochemical parameters in the solutions of ethanol in water is presented. As can be seen from Fig.1 for the dependence of partial molar volume /curve 1/ [68 - 70], position of the maximum of the luminescence band of salicylic acid / 2 / [71], heat of mixing / 3 / [67], isothermal compressibility / 4 / [72,73] and isoviscosity coefficient of selfdiffusion of water / 5 / [74] the extrema can be observed in the concentration range 0.05 - 0.1 k_{mol} and the differences of the concentrations of the maximum points are connected with the different measurement temperatures. In Fig.2 the results of our measurements of ultrasound velocity at 25°C /curve 1/ and the shifts of the valence - deformation band of absorption of water $\nu_2 + \nu_{OH}$ with the maximum at

5180 cm^{-1} /curve 2/ are presented. As can be seen from Fig.2 the ultrasound velocity increases at first, reaches a maximum at the concentration k_{mol} = 0.106 and then decreases. On the curve of $\Delta\nu_{max}$ as a function of alcohol concentration an increase of the shift of the studied absorption band in the concentration range 0 - 0.1 k_{mol} is observed and for the higher concentrations this shift is constant and independent of alcohol concentration up to $k_{mol} \approx 0.4$ [75].

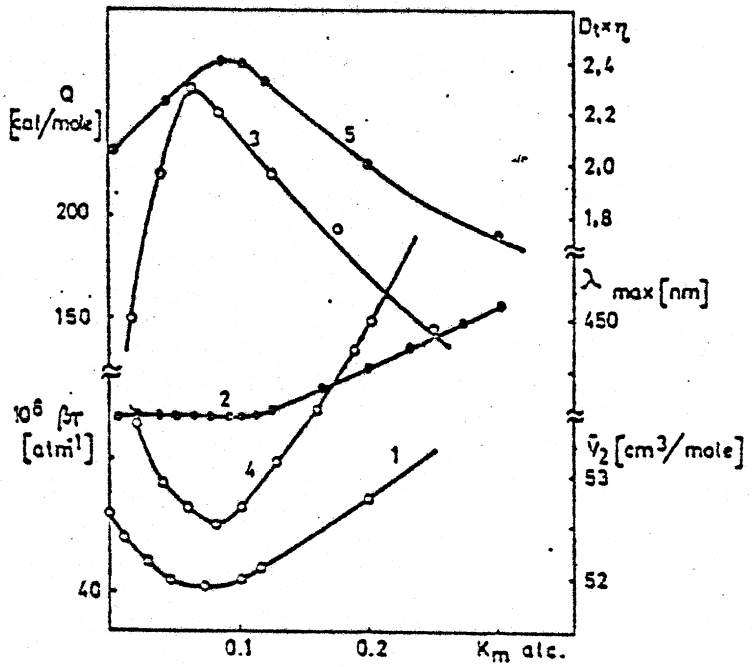


Fig.1.

The dependence of partial molar volume /1/, position of the maximum of the luminescence band of salicylic acids /2/, heat of mixing /3/ isothermal compressibility /4/ and isoviscosity coefficient of selfdiffusion of water /5/ on the concentration of alcohol for the ethanol-water system .

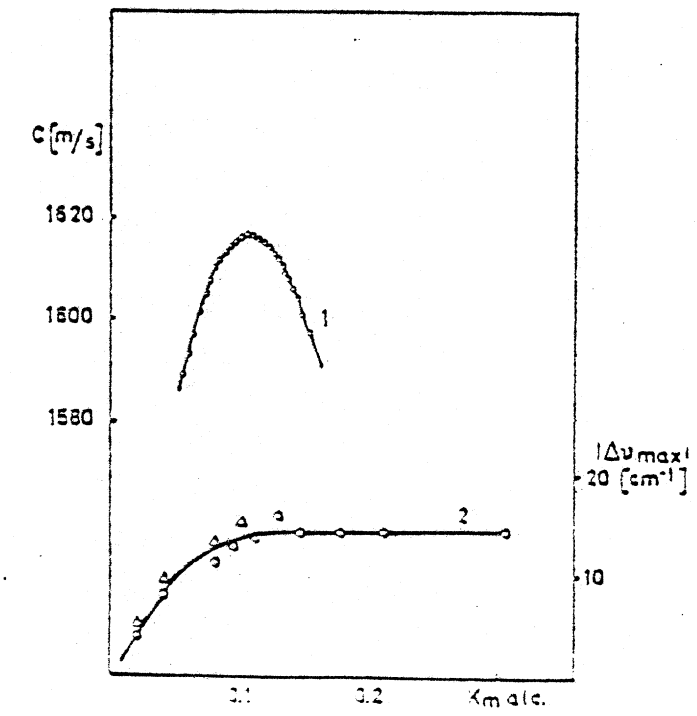


Fig.2.

Ultrasound velocity /1/ and the shift of the valence-deformation band of absorption of water with the maximum at 5180 cm^{-1} /2/ versus k_{mol} of alcohol for the ethanol-water system.

The previous spectroscopic infrared investigations [75 - 78] showed that the stabilization of the structure of water by non-electrolyte molecules is connected with the increase of stability of hydrogen bonds between water molecules. This effect is reflected in the shift of absorption bands in the direction of lower frequencies. The observed order of water is due to influence of non-polar groups of non-electrolyte molecules on water; around the non-electrolyte molecules associated molecules of water called non-polar group solvates appear. This type of solvation is defined as second order solvation [45].

Mathematical analysis of the shift of the absorption band at 5180 cm^{-1} assuming Gaussian character of the contiur of this band allowed the determination of the mean number of water molecules in the non-polar group solvate. For ethyl alcohol it equals 6 ± 1 [75] - 7 ± 1 [77,78] at 20°C and it decreases with an increase in temperature. The decrease of hydrophobic hydration of non-electrolytes with temperature increase was suggested also in the investigations of isothermal compressibility of non-electrolyte solutions [73]. The authors of these investigations think that hydrophobic hydration is characterized by the slope of curves β_T i.e. by values of $\left(\frac{\partial \beta_T}{\partial x}\right)_{x \to 0}$ /this is an analogical assumption to that of Allam and Lee [14] for electrolyte solutions/. The increase of this value corresponds to the increase of hydrophobic hydration of non-electrolyte molecules. Analysing the course of the dependence of ultrasound velocity on ethanol concentration and temperature similar conclusions can be drawn. As is known the maxima on the ultrasound velocity curves /minima on the compressibility curves/ shift in the direction of lower concentrations with temperature increase. This is caused by a decrease of the "open-work" structure of water with increase in temperature. The amount of empty cages /"holes"/ which can be occupied by alcohol molecules decreases and the amount of "free" water molecules increases. Assuming that at 0°C all the water molecules occur in an ice-like skeleton it can be pressumed with great probability that the concentration of the ultrasound velocity maximum at 0°C defines the amount of molecules surrounding the non-polar molecule. Due to the impossibility of defining this concentra-

tion at 0°C with satisfactory accuracy /the error of velocity measurement ought not to exceed 2 cm/s / the ultrasound velocities at higher temperatures were determined.
In Fig.3 the results of our measurements in ethanol-water solutions in the temperature range of 5 - 35°C are presented.

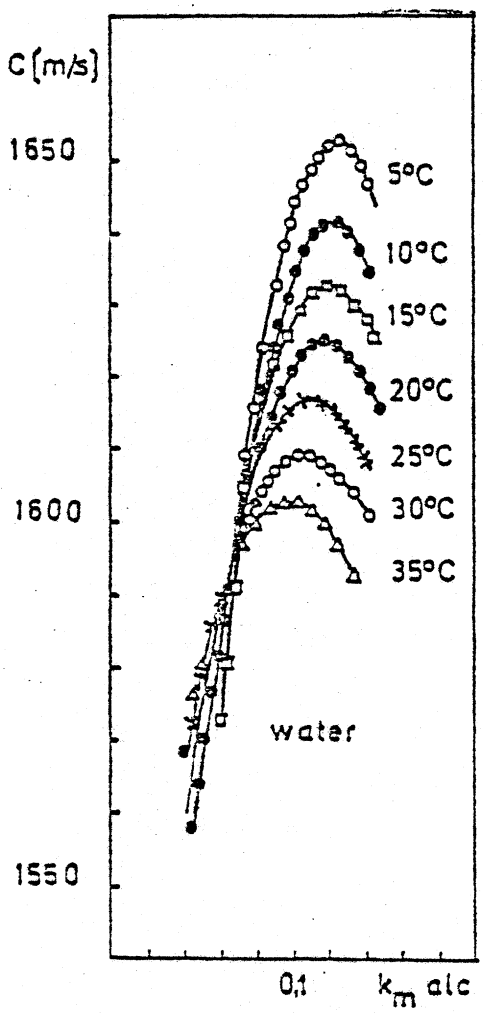


Fig.3.
Ultrasound velocity versus k_{mol} of alcohol in the temperature range 5 - 35°C for the ethanol -water system.

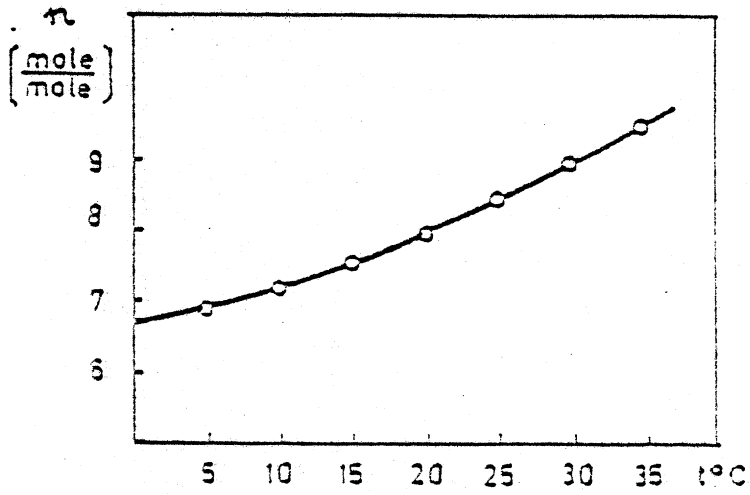


Fig.4.
The numbers of water molecules per one alcohol molecule as a function of temperature at maximum point of ultrasound velocity.

As can be seen in Fig.3 the ultrasound velocity maximum shifts in the direction of lower concentrations of alcohol. The numbers n / mole/mole / of water molecules per one alcohol molecule were determined at maximum points in the investigated temperature range and are graphically presented in Fig.4. Extrapolating to 0°C temperature the value of n = 6.6 was obtained. The result obtained is in good agreement with the results of infrared measurements. Theoretically at 0°C n should be equal to 6 for the ideal crystal lattice of ice /octahedral structure/. However, considering the occurrence of defects in water structure /Frenkl and Schottky defects/, the amount of empty cages is less and $n > 6$.

Forslind [79] calculated that in liquid water at 0°C 9 per cent of the lattice nodes are unfilled and the amount of molecules in interstitial positions equals 16 per cent of the total amount of molecules in the system. From the calculations which take into consideration the experimental value of n and also its theoretical value it is possible to draw analogical conclusions.

The amount of water bound in the lattice at 20°C calculated on the basis of the data obtained /Fig.4/ is equal to 76.5 per cent and agrees well with the data calculated by Nemethy and Scheraga [80] using statistical thermodynamic analysis of the changeable groups of liquid water molecules model.

If we accept n real to be 6.6 , then at 20°C the amounts of bound water /83%/ and "free" water /17%/ are in good agreement with analogical values given by Nomoto [81].

The concentration at the maximum point of ultrasound velocity at 0°C determines therefore the amount of water molecules which occurs in the solvates of a non-electrolyte molecule. As temperature increases the deformations of hydrogen bonds between molecules of water increase and therefore the coordination numbers increase and the numbers of "holes" /cages/ decrease. This leads to, in consequence, weakening of the stabilization effect. A similar opinion is represented by Hertz and Zeidler [45]. On the curves of ultrasound velocity this process reflects itself in the decrease of velocity value with temperature increase for maximum points and in broadening

of the curves. By extrapolating the curve of Fig.4 to a temperature of 80°C it is possible to approximate the percentage amount of water involved in the solvates of non-electrolyte molecules. From the calculations it appears that at 80°C less than 10% of the water molecules occurs in bound form. Therefore, it is not strange that at this temperature a maximum for the velocity curves in the water-alcohol system does not occur.

The results presented confirm the legitimacy of the accepted model. The curves of ultrasound velocity vs. alcohol concentration describe in a real way the structure of alcohol-water solutions. The maximum of ultrasound velocity defines the alcohol concentration at which all of the cages of the undestroyed structure of water are accupied by alcohol molecules. The increase of non-electrolyte concentration to above the maximum value causes the break up of the primitive structure of water, the degree of order of the solution decreases and as a result of this the ultrasound velocity decreases.

As mentioned previously the addition of any solute to the alcohol-water mixtures causes a shift of the peak of the parabola in the direction of lower concentrations of alcohol. This is caused either by break up of the water structure and the binding of the water molecules freed in this way by the solute or by accupation by the solute molecules of part of the "holes" in the water structure. In both instances the amount of empty cages occupied by alcohol molecules decreases and the alcohol concentration at which the ultrasound velocity reaches a maximum is smaller.

The difference between the concentration at the velocity maxima of the alcohol-water solution of the solute and the concentration of the reference solution is caused by hydration /I or II order/ of the solute.

The amount of hydration water per amount of grams of the solute W_x can be calculated from the following formula /2/:

$$W_x = W_1 - \frac{W_0 A_1}{A_0} \qquad /3/$$

Apparatus

The condition for obtaining reproducible and authentic results of measurements of hydration numbers from ultrasound velocity measurements is the precise determination of the maximum point.

In our paper [82] it was shown that in the case of not too highly concentrated solutions /lower than 5%/ the maximum point should be determined with an accuracy up to 0.2 ml of alcohol which corresponds to the ultrasound velocity changes of 1 - 2 cm/s. With the aim of carrying out the measurements with such a high accuracy the measurement system described below was used. The block diagram of this system is shown in Fig.5.

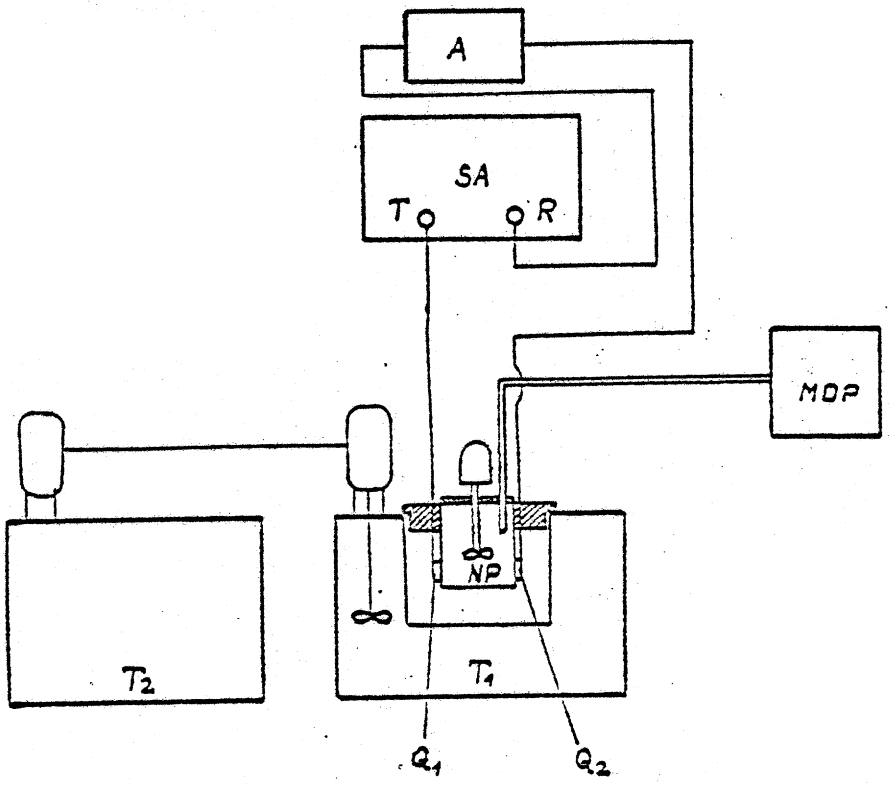


Fig.5. Block diagram of the apparatus used for the measurement of the velocity of ultrasonic wave.

SA - ultrasonic wave velocity meter of the type SA 1000, developed by the Institute of Fundamental Technological Research, Polish Academy of Sciences, Warszawa, which enables a measurement of relative velocity to be made with an accuracy of up to 1 cm/s, A - high frequency resistance attenuator, MDP - small-dimension metering pump, type 355 A developed by UNIPAN, Warszawa, which monitors the flow of ethanol into the measuring vessel with an accuracy of up to 0.02 ml, T_1 and T_2 - thermostat systems developed by UNIPAN, Warszawa, which ensure constant temperature in the measuring vessel with an accuracy of 0.002°C, and NP - measuring vessel with agitator and transmitting and receiving transducers Q_1 and Q_2.

Such a good accuracy of the measurements of relative values of ultrasound velocity was obtained thanks to a very sensitive ultrasound velocity meter, construction of suitable measuring vessels immersed in the thermostat and to good thermostating with an accuracy up to ± 0.002°C. A digital velocity meter "sing around" of the type SA 1000 developed by the Institute of Fundamental Technological Research, Polish Academy of Sciences, Warszawa, was used for ultrasound velocity measurements. The change of the last figure on the wave meter display gives, depending on the measuring vessel used, the change of velocity of the order of 0.5 - 1 cm/s. A cylindrical measuring vessel of stainless steel which has a small coefficient of expansion /50 mm diameter/ was immersed at a constant level in the thermostat. The thermostat had a 25 dcm^3 capacity, double walls and was powered and controlled by a temperature regulator of the type 650 and power unit of the type 651 developed by UNIPAN, Warszawa. The regulator and the power unit are part of a microcalorimeter produced by that firm. The regulator and the power unit are automaticlly controlled by a resistance platinum sensor of the type 2100 s-3 wire - 100 om of English production. The temperature in the measuring vessel was controlled by the same kind of resistance sensor with an accuracy of ± 0.002°C. In addition the temperature in the thermostat was controlled by use of the precision Beckman's thermometer with an accuracy up to 0.005°C. The thermostat was cooled by water from a second thermostat in which the temperature of the thermostating water was 2 - 3°C lower than the temperature of the water in the main thermostat. With the aim assuring stable work of the "sing-around" velocity meter, the wave meter and the regulator, the environment temperature was constant / 21 ± 1°C /. The measurements of hydration numbers for monocarboxylic acids, amino acids, amides, saccharides, peptides and macromolecules were carried out at 25°C.

Results

1. Determination of hydration numbers of monocarboxylic acids, of aminoacids and of amides in aqueous solutions.

The hydration number n_h [mole/mole] of the following monocarboxylic acids in aqueous solution was measured at the

concentration range of 3 to 10%: acetic acid, propionic acid, n-butyric acid and acrylic acid. The n_h value was also determined for such aminoacids and amides as: glycine, α- and β-alanine, α-aminobutyric acid, acetamide, propionamide and acrylamide at the same concentration range of the aqueous solution. The aim of these measurements was to determine the contribution of the functional groups -COOH, $-NH_2$, $-C(=O)-NH_2$, $-C(=O)-$ to the total hydration of the molecule and to confirm, the previously suggested thesis [83] concerning the hydration of hydrophibic groups as well as to determine the contribution of these groups in the total hydration of a molecule.
The results are presented in Tables I - III.

Table I. n_h [mole/mole]

C %	CH_3COOH	C_2H_5COOH	C_3H_7COOH	$CH_2 = CHCOOH$
3	4,8	8,0	11,5	5,8
5,7	4,2	8,4	10,3	6,2
6	4,9	8,3	9,6	6,8
8	5,0	8,0	10,2	6,0
10	4,7	8,5	10,1	6,3

Table II. n_h [mole/mole]

C %	glicyne	α-alanine	α-amino butyric acid	dl-valine	β-alanine	L-Lysine
3	5,7	7,6	10,2	13,5	6,9	13,6
5	6,1	8,2	10,2		6,7	13,0
6	6,0	7,6	9.75		6.7	13.2
8	6.0	8.0				12.8
10	5.7	7.6				

Table III. n_h [mole/mole]

C %	CH_3-CONH_2	$C_2H_5CONH_2$	$CH_2=CH-CONH_2$
3	3,0	5,0	4,2
7	2.5	5.2	4.0
8	2.5	5.5	3.8
10	3.0	5.5	3.7

2. Determination of hydration numbers of saccharides.

Measurements of hydration numbers [mole/mole] using ultrasonic method were performed in solutions of saccharides such as: glycose, mannose , rhamnose, arabinose, lactose and sucrose within a concentration range of 3 - 8%.
The results of the measurements are collected in table IV.

Table IV. n_h [mole/mole]

C %	glycose	mannose	rhamnose	arabinose	lactose	sucrose
3	4.7	4.8	4.0	4.0	9.1	9.0
5	5.2	4.8	3.8	4.0	9.5	9.2
6	5.2	5.1	3.8	3.7	8.8	9.1
8	5.1	5.1		3.5	9.5	8.9
20	4.8					

3. Determination of hydration numbers of oligopeptides.

Table V. n_h [mole/mole]

C %	diglycine	triglycine	glycil-alanyl glycine
3	9	13.4	13.5
5	8.8	13.0	
6	9	12.0	
8	8.5		

4. Determination of hydration numbers of macromolecules in aqueous solutions.

4.1. Polyethylene glycol

The hydration numbers of several polyethylene glycols in aqueous solutions were determined at the concentration range of 1.4 - 14.5%. The molecular weights of the glycols were: 400, 1500, 2000, 15000 and 20000. All were from BDH Chemicals LTD. These measurements were carried out in order to determine the optimal concentration range for the measurements and to

evaluate the relation between the hydration number and the molecular weight of the molecule .
The results are presented in Table VI. The relation between the ultrasound velocity and the concentration of ethanol in the aqueous solution is exemplified on Fig.6 for the 400 and 20000 polyethylene glycols.

Table VI.

polyethylene glycol	C % 1.43	2.86	5.71	8.71	11.43	14.29
400	0.511	0.767	0.894	1.022	1.149	1.175
1500	0.511	0.767	1.022	1.022	1.149	1.124
2000	0.511	0.511	0.894	1.107	1.149	1.175
15000	0.511	0.767	1.149	1.022	1.149	1.226
20000	1.022	1.022	1.149	1.072	1.149	1.226

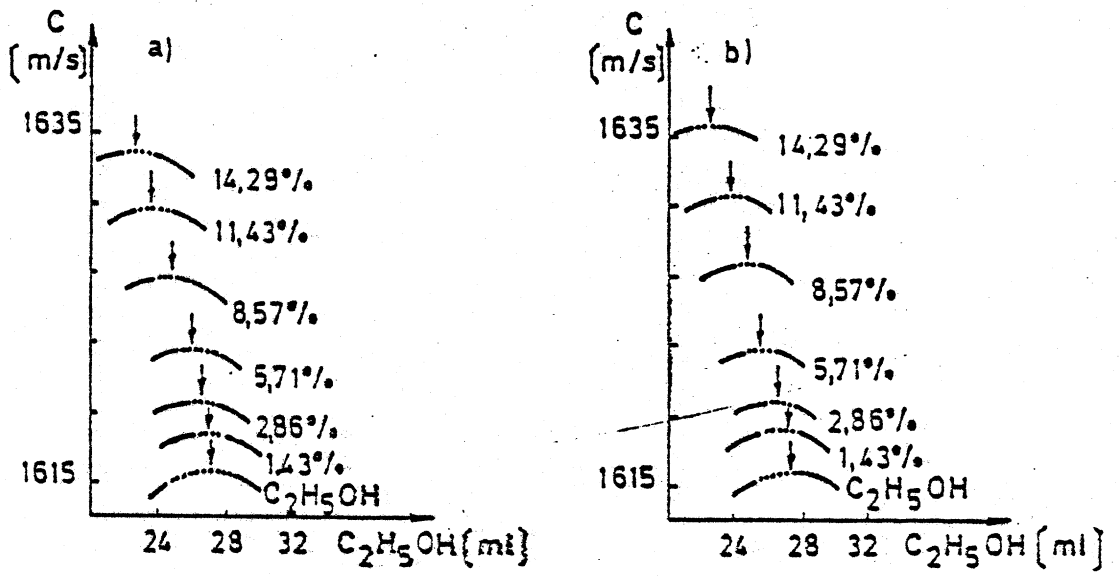


Fig.6. Ultrasound velocity as a function of ethanol concentration for ethylene polyglycol with molecular weight 400 and 20000.

4.2. Dextrane

The hydration numbers of two dextranes /mol. weight 40,000 and 500.000/ in aqueous solutions were determined at the concentration range of 2 - 15%. The two dextranes were chosen so as to differ markedly in thier molecular weights and it was expected to find out whether the hydration depends on the molecular weight of such types of compounds. The results are gathered in Table VII. The hydration numbers expressed in moles of H_2O per mole of the $-C_6H_{10}O_5-$ unit are given in positions 2 and 4 of the Table.

Table VII.

dextrane		C %	2.0	4.3	5.7	7.14	11.4	12.8	15.0
500.000	1	n_h cm^3/g	0.511	0.340	0.383	0.357	0.287	0.283	0.279
	2	n_h m/mmon	4.6	3.1	3.4	3.2	2.6	2.5	2.5
40.000	3	n_h cm^3/g	0.511	0.340	0.383	0.360	0.319	0.340	0.325
	4	n_h m/mmon	4.6	3.1	3.5	3.2	2.9	3.1	2.9

4.3. Polyacrylamide

Polyacrylamide of varying molecular weight was prepared from 10 - 15% aqueous solutions of acrylamide. The polymerisations were carried in the presence of perhydrol in the amount of 0.5 to 1 ml per 100 ml of the solution.
The reagents were activated by submitting the reaction mixture to the action of 22 kc ultrasonic waves of 3 W/cm^2 intensity for a period of 15 - 30 minutes. After sonification the solutions were air-freed by bubling through a stream of nitrogen. The solutions were then kept in tightly stoppered flasks up to the end of the polymerisa tion processes which lasted two to twenty hours depending upon the concentration of acrylamide and perhydrol and duration of sonification.
So obtained samples were diluted with water and the molecular weights of polyacrylamide were determined by applying the

light-scattering method.
The hydration numbers of the samples were determined as previously described. The results are presented in Table VIII. The hydration numbers expressed in moles of H_2O per mole of the $-CH_2-\underset{|}{CH}-C(=O)-NH_2$ unit are given in positions 2,4 and 6 of the Table. For comparison the values of hydration number determined for acrylamide at the same concentration are given in position 7.

Table VIII

polyacrylamide		C. %	2.0	3.0	4.0	5.0	n_h^{av}
32.000	1	n_h cm^3/g	1.478	1.135	1.006		
	2	n_h m/m mon	5.8	4.4	3.9		4.15
40.000	3	n_h cm^3/g	1.127	1.018	0.987	1.189	
	4	n_h m/m mon	4.4	4.1	3.9	4.6	4.25
200.000	5	n_h cm^3/g	0.930	1.000	0.914		
	6	n_h m/m mon	3.7	3.9	3.6		3.7
acrylamide	7	n_h m/m	4.3	4.2	4.0	3.9	4.1

Discussion

1. Carboxylic acids, amino acids, amides and oligopeptides

The purpose of hydration number measurements for carbocylic acids, amino acids and amides was to determine the contributions of the particular functional groups occurring in these compounds to total hydration and to confirm the previously suggested thesis [8] on the hydration of hydrophobic groups. The results obtained confirmed the legitimacy of this thesis. For all investigated compounds the hydration numbers increase with the increase of hydrocarbon chain length and the average number of molecules of hydration water per group $-CH_2-$ equals 2. In order to determine the contribution of the functional groups to total hydration, certain assumptions were made:

1/ In the investigated concentration range the degree of dissociation of monocarboxylic acids is so small that the presence of ions in the solution is not taken into consideration and the acid molecules are treated as polar molecules. This assumption included also amino acids.

2/ The alkyl groups directly bound with carboxylic groups are not hydrated.

The legitimacy of the second assumption is obvious if the hydration numbers of amino acids are compared.
If the alkyl groups directly bound with the carboxylic group were hydrated, the difference between hydration numbers of α- and β-alanine would not be observed. However, on the basis of the presented data it can be seen that the substitution of the $-NH_2$ group in the β-position decreases the hydration number relative to the α-isomer. Therefore, the hydrocarbon group in the β-position makes a basic contribution to the total hydration of the $-C_2H_5$ alkyl group.
The hydration numbers of functional groups calculated on the basis of the above assumptions are presented in Table IX.

Table IX.

group	n_h
-COOH	5 , 3 - 4 [1] , 2 [84]
$-NH_2$	1 , 1 [1] , 1 [85]
$-CH_2=CH-$	1 - 2
C_2H_5-	2 - 3
C_3H_7-	5 - 6
$-CH_2-$	~2, 2 [83] , 1 [84]
=C=O	2, 2 [1]

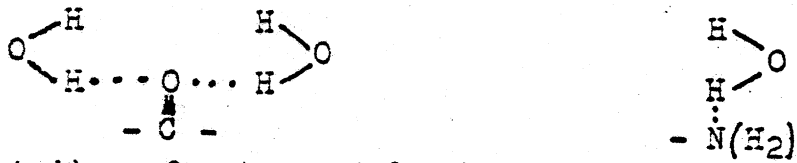
With the aim of confirming the legitimacy of the assumptions which were made and of the conclusions which were drawn the hydration numbers of certain oligopeptides such as: diglycine, triglycine, glycyl-alanyl-glycine and the amino acid l-lysine were compared with the hydration number values calculated on

the basis of the contributions of the particular groups occurring in these compounds. It was assumed that the -NH- group which is present in the peptide group $-C\overset{O}{\underset{NH-}{<}}$ is hydrated by one water molecule similarly to the $-NH_2$ amine group. Comparitive data are given in Table X.

Table X.

	n_h measur.	n_h calc.
diglycine	9	9
triglycine	13	12
glycyl-alanyl-glycine	13.5	14
l-lysine	13	13

As can be seen from the above results a good agreement of the hydration numbers experimentally determined and those which were calculated on the basis of our assumptions was achieved. The investigations make it possible to draw certain conclusions about the way in which the water molecules are bound by the particular groups. It seems possible that the water molecules are bound by hydrogen bonds with polar groups through the free electron pairs of the nitrogen and oxygen atoms:



The binding of water mainly through the free electron pairs of nitrogen and oxygen atoms would explain in some way the small hydration number of the $-NH_2$ /or -NH-/ group and the large hydration number of the -COOH group. On the other hand, alkyl groups in carboxylic acids are most probably hydrated likewise as non-electrolyte molecules /II order hydration/. The creation of weak hydrogen bonds between hydrogen of alkyl groups and oxygen of water can not be excepted /particularly in the case of amino acids/.

2. Saccharides

As can be seen from Table IV the hydration numbers of saccharides depend on the amount of hydroxyl groups occurring in a given saccharide. These numbers indicate that one hydroxyl

group of saccharide is hydrated by one water molecule. The results obtained are in good agreement with the results given by Shiio [10,11] and correspond with the data of other methods: differential scanning calorimetry [86] , dielectric relaxation NMR [41,86] and equilibrium point of solution [87,88].
Comparing the results obtained by the use of these different methods it should be remembered that different assumptions are made in these methods, that the measurement temperatures are different /up to subzero/ and that the concentration of solutions are mostly high. Therefore, there is no complete agreement of hydration number values obtained using the different methods.
The conclusion of Harvey et. al. [90] about the binding of two water molecules by the -OH group of saccharide drawn on the basis of the assumption that the hydroxyl groups of saccharide and alcohol affect the water in a similar way as in pure alcohol can not be valid in view of the facts presented above about the stabilizing influence of alcohol on the water molecules.

3. Macromolecules

The hydration number measurements in solutions of macromolecules such as: polyethylene glycol, dextran and polyacrylamide with the different molecular weights were to examine the influence of size of macromolecules on their hydration value.
In case of polyethylene glycols we wanted to optimise the concentrations of macromolecules for which this method gives reproducible results. As can be seen from Table VI in the concentration range 1.4 - 5.7 g/100g H_2O the overall amount of bounded water was determined from the parabolic shifts and gave values within the range 0.8 and 5.0 ml H_2O. The smallest addition of alcohol which causes noticeable changes in the velocity maximum of the parabola is 0.2 ml, which gives an equivalent amount of bounded water equal to about 0.6 ml. In connection with this it must be stated that if the total amount of bounded water for a given amount of solute is lower than 6 ml the significant error is greater than 10 per cent and the results cannot be taken as accurate.
Within the concentration range 1.4 - 5.7 g/100g H_2O the significant error exceeds 10 per cent, and for the lowest investigated concentrations approaches 60 per cent, and consequently

for this concentration the results cannot be considered as significant.

In order to substantiate our results, measurements of longitudinal relaxation time T_1 were carried out on aqueous solutions of polyethylene glycols in the concentration range given above.

The investigations were performed on an impulse spin-echo apparatus at a frequency of 25 MHz using the method of three impulses 180-90-180 at constant temperature 22°C. The relationship between relaxation time T_1 for aqueous solutions of macromolecules and their concentration is expressed by the following equation: [43, 91 - 93]

$$\frac{1}{T_1} = \frac{1}{T_{1w}} + K \cdot c$$

where: T_1 - represents the longitudinal relaxation time for solution, T_{1w} - is the relaxation time for pure water, c - is the concentration of solute and the K value is proportional to the amount of water bound with the molecules of the dissolved substance. Water in hydration shells posseses lower mobility when compared with free water, unbounded with macromolecule and therefore relaxation process of unbounded water macromolecules is considerably quicker than that of water bounded with macromolecules of dissolved substance. If we assume that the exchange between free water macromolecules and bonded ones is very fast, we may observe the shortening of the relaxation time T_1 for the solution in relation to pure water. The results of longitudinal relaxation time T_1 messurement for aqueous solutions of polyethylene glycols with molecular weights 400, 1500, 2000, 15000 and 20000 within the concentration range 4 - 10% showed that the relaxation time is independent of the polyethylene glycol chainlength. It is thus concluded that the amount of bound water is independent of the polyethylene glycol molecular weight and only dependent on its solution concentration. It is in good agreement with the results of ultrasonic measurements. Hydration number independence of the molecular weights of polymers was also shown in case of dextran with the molecular weights

40.000 and 500.000 and polyacrylamide 32.000 and 40.000. However, the hydration number values for polyacrylamide 200.000 were little lower which suggests that in aqueous solution polyacrylamide with such a high molecular weight forms aggregations or microgels. This conclusion was partially confirmed by the light scattering measurements. Number of water molecules per one group $-C_6H_{10}O_5-$ in dextran with the molecular weights 40.000 and 500.000 is 3 and corresponds with hydration number of saccharides /Table IV/ and given by Shiio [11]. Number of water molecules per one $-CH_2-\underset{|}{CH}-C\overset{O}{\underset{NH_2}{<}}$ group in polyacrylamide with the molecular weights 32.000 and 40.000 equals 4 and is the same as hydration number of monomeric acrylamide. It is thus concluded that water molecules are bound with the help of hydrogen bond by polymer in the same way as by monomeric molecules and conformeric transformations of polymeris with coil-ball type or medial conformations do not cause changes of amount of water bound with polymer.

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Part II. Investigation of hydration of casein and its fractions

The hydration of casein and its fractions and the hydration of dry milk solids was determined by the use of Yasunaga's method which was previously described. The dry solid mass of not fat milk was obtained using the weight method by evaporation of milk at 102°C until a constant dry mass was achieved. Casein was prepared using two methods. The end product of one of these methods was the calcium form of casein and of the other was the sodium form. The α_s-, β- and $\varkappa$-caseins were obtained from the sodium form.

1/ The preparation of casein I /calcium form/

The total casein fraction of unpasteurized skimmed milk was precipitated by adding the required amount of 1N HCl to obtain pH of 4.6. The precipitated casein was centrifuged off and washed with an excess of distilled water, dialyzed and liophylized.

2/ The preparation of casein II /sodium form/

Casein II was prepared from not fat milk by adding 1N HCl solution in order to obtain pH = 4.6 [1]. The casein which precipitated was separated from the whey and rinsed twice with distilled water. The protein precipitate was transformed into a suspension and 1N NaOH solution was added to it in order to dissolve the casein. The casein was again precipitated by adding 1N HCl solution until pH 4.6 was reached and then after separating the precipitate it was rinsed with distilled water three times. The casein precipitate was dissolved by the addition of 1N NaOH and then was dried by liophilization.

3/ The preparation of the α_s-casein fraction

The α_s-casein fraction was prepared from casein II by dissolving it in 6.6 M urea solution and then it was diluted to a concentration of 3.3 M at pH 4.6 - 4.8 [2]. The precipitate complex of α-casein was separated from the solution and again dissolved in a 6.6 M urea solution. After dilution of the solution to a concentration of 3.3 M urea, the preci-

pitate of α-casein was separated and rinsed several times with distilled water. In the next stage the α-casein complex was dissolved in water with the addition of 1N NaOH solution until pH 7.2 was reached, it was next cooled to a temperature of 2 - 4°C and the α_s-casein was precipitated by adding 4M $CaCl_2$ solution to the concentration of 0.4 M. The α_s-casein precipitate was separated from solution, dissolved in 5M urea solution and next diluted to a concentration of 1M at pH 4.6. This α_s-casein fraction was dissolved in distilled water by the addition of 1N NaOH until pH 7.2 was reached and next it was precipitated by adding 1N HCl to pH 4.6. This process was repeated three times. In the final stage the fraction was dissolved in water at pH 7.5 and was dried by liophilization.

4/ The preparation of the β-casein fraction

The β-casein fraction was prepared from casein II by dissolving it in 6.6 M urea solution and next by diluting to a concentration of 3.3 M at pH 4.6 3 . The precipitate was separated and the solution containing β-casein brought to pH 4.9 , diluted threefold with distilled water and heated to 30°C. The β-casein precipitate was separated from solution and purified by dissolving twice in 3.3 M urea solution and precipitated at pH 4.9. In the final stage the fraction was dissolved in water at pH 7.5 and dried by liophilization.

5/ The preparation of the $\varkappa$-casein fraction

The $\varkappa$-casein fraction was prepared from casein II by dissolving it in 6.6 M urea solution and by the addition of 7N H_2SO_4 solution to pH 1.3 - 1.5 [4]. After 2-4 hours the precipitate which had formed was removed and $\varkappa$-casein was precipitated by adding $/NH_4/_2SO_4$ to the filtrate to a concentration of 1 M. The precipitate was separated from the solution, dissolved in water with the addition of 1N NaOH to pH 7.5 and dialyzed. Then, 1M NH_4COOH in a 75% ethanol solution was added to the $\varkappa$-casein solution until the precipitate was obtained. The precipitate was separated from the solution, rinsed with distilled water and next purified by dissolving

twice in water with the addition of 1N NaOH and precipitation at pH 4.6. In the final stage the fraction was dissolved in water at pH 7.5 and dried by liophilization.

Results

The results obtained from the hydration measurements / ml/g of casein and its fractions are collected in Table I. The presented numerical data are the average of two measurements for each concentration and the error of measurement did not exceed 0.1 ml/g.

Table I

h [ml/g]

casein I /calcium form/	c	1,5 %	2,8 %
	h	0,38	0,40
casein II /sodium form/	c	2,8 %	4,3 %
	h	0,62	0.60
α_s-casein	c	2.8 %	4.3 %
	h	0.49	0.57
β-casein	c	2.8 %	4.3 %
	h	0.70	0.65
$\varkappa$-casein	c	2.8 %	4.3 %
	h	0.65	0.62
solids-not-fat skimmilk components		0.36	0.42

Discussion

As can be seen from Table I casein I and the dry milk solids have almost identical numerical hydration values. The fresh not fat milk used in the measurement contained averagely 8.4 % of dry solids. The approximate contant of dry solids was as follows: 30% casein /α; β; $\varkappa$/, 5% of other protein compounds, 60% lactose /α; β-/ and 5% of mineral salts, vitamins and other components. Casein and lactose make up therefore 90% of the dry milk solids. As can be seen from the data presented in the I part of this report, the hydration of lactose is numerically equal to the hydration of casein. It is not

strange therefore, that the results of the measurements for casein and for dry milk solids are the same. The numerical hydration values for casein and solids-not-fat skimmilk are comparable to the literature data [5]. The hydration of casein II occurring in the sodium form is greater than that of casein II and equals ~ 0.6 ml/g. Simultaneously a better solvation of casein II is observed. The increase of bound water is caused by the greater ability of sodium caseinate to dissociate in comparison to calcium caseinate. Ionic forms of macromolecules will in every case bind a greater amount of water than non-ionic forms. The comparison of the hydration of α_s, β, $\varkappa$-casein fractions and casein II leads to the conclusion that also in this case the numerical hydration values are almost identical. Some small differences can be noticed between $\varkappa$- and β-casein and between α_s-casein. These observed differences lie however within the measurement error /~ ± 0.1 ml/g /. The results should be accepted as probable because, according to Rose et al. [6-8], into the micelle contents of casein enters statistically about 50% α-casein, 35% β-casein and 15% $\varkappa$-casein. Accepting that the hydration of total casein is an additive value relative to the hydration of its particular fractions, it seems obvious that the casein fractions have numerical hydration values similar to the hydration of total casein. The qualitative differences in the hydrations of α_s, β-and $\varkappa$-casein would indicate that the α_s-casein forms the internal part of the micelle and on the other hand the fractions of β- and $\varkappa$-casein which have greater affinity for water appear rather in the external sphere of the micelle. The above conclusion agrees with the model assumptions of casein micelle given by Payen and Rose [6,7].

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Part III. Model investigations of the detergent-casein interactions

The nature of the interactions between proteins and amphiphatic substances is essential for many conditions of importance in biological systems.
The prevailing investigations of detergent-protein [1,2], lipid-synthetic polipeptides systems [3] and systems of polymer-detergent show that the specific interactions are formed between protein /or polymer/ and detergent. The effect of these interactions is the binding of a part of the detergent molecules by a macromolecule. This causes visible changes in the concentration dependences on the surface tensions, the conductivity, the viscisity etc. and can be observed in the IR spectra. Detergent-protein interactions have mainly been studied with the use of synthetic anionic alkyl sulphates and bovine serum albumin. Less is known about the interactions between cationic detergents and proteins and between proteins and neutral detergents. The interactions between detergents and casein have not yet been studied. This study presents investigations of the bindings of casein with different types of detergents: cationic, anionic and non-ionic. The results of these investigations can be helpful in the understanding of the mechanism of the interactions of milk lipids with the casein micelle and of the influence of the quantity and the types of lipids on the stabilization of the micelle and consequentially on the stabilization of milk and its products. The investigations were made by means of three measurement methods:equilibrium dialysis, surface tension and ultrasound velocity.

1. Interaction of casein with cationic and non-ionic detergents studied by equilibrium dialysis method and surface tension method

This study presents investigation of the binding of cationic detergents with different hydrocarbon chain length such as alkyl pyridinium bromides to casein and binding of non-ionic detergents such as decylglucoside to casein tested by means of equilibrium dialysis method and surface tension method.

1.1. Materials and methods

The following reagents, obtained from the indicated sources were used: fat free casein /BDH, England/; myglobin, 2 x cryst chymotrypsinogen, 6 x cryst., β -lactoglobulin, 3 x cryst., ovalbumin, 5 x cryst., and bovine serum albumin, cryst., /were all purchased from Koch-Light, Colnbrook, England/. Blue dextran and Sephadex G-150 were purchased from Pharmacia, Uppsala, Sweden. The n-alkylpyridinium bromides were synthesized from ultrapure alkyl bromides, purchased from Eastman--Kodak, Rochester N.Y. USA, and pyridine in alcohol solution according to the method of Czarnecki and Kowal [6] . The alkylpyridinium bromides were recrystalized six times from boiling acetone and stored in a desicator over solid hydroxide The decyl β -D-glucoside was synthesized from spectraly pure decanol /from BDH, England/ and glucose according to Noller and Rockwell's method [7]. DeG had been recrystalized three times from ethylacetate and the product gave elementar analysis. Hygroscopic DeG was stored in a desicator over solid sodium hydroxide.

Initial purification of casein. The purification procedure was carried out using a column /5 x 100 cm/ of Sephadex G-150 equilibrated with phosphate buffer, pH 6.8, ionic strength 0.1, containing 0.02% NaN_3 at 5°C. The protein was dissolved in phosphate buffer and stirred for 1 h and ultracentrifuged at 100000 x G for 30 min. The supernatant was applied to a column and the elution was carried out with the same buffer. Fractions of about 6 ml were collected at a flow rate of about 60 ml/h. Two peaks of protein were obtained /casein I and II/. Fractions containing casein II were pooled and have been used in further experiments.

Abbreviations used: OPB - octylpyridinium bromide, DePB - - decylpyridinium bromide, DPB - dodecylpyridinium bromide, TPB - tetradecylpyridinium bromide, DeG - decylglucoside, CMC - critical micelle concentration.

Analytical gel filtration on Sephadex G-150. A column /1.4 x x 100 cm/ was equilibrated with phosphate buffer, pH 6.8, ionic strength 0.1, containing 0.01% NaN_3. 1 ml of the prepared casein II /see above/ was applied to the column. The column was calibrated with the following markers: myoglobin, chymotrypsinogen, β-lactoglobulin, ovalbumin and bovine serum albumin. Samples of each protein /10 mg/ and Blue dextran were dissolved in 1 ml of the equilibrating buffer and layered on the top of the column. The elution was carried out with the same buffer at a flow rate of 6 ml/h. The concentration of Blue dextran and proteins were detected by using flow absorptiometer Uvivord /Sweden/ at 280 nm.

Equilibrium dialysis. Cationic detergent binding measurements were carried out by the equilibrium dialysis method using a shake Teflon dializer consisting of 32 units. The equilibrium dialysis was carried out in phosphate buffer, pH 6.8, ionic strength 0.1, containing 0.01% NaN_3, at room temperature. Non-ionic detergent binding measurements were carried out in the same buffer but without NaN_3 at 10°C. The Visking membrane was placed in boiling water for one hour and rinsed thoroughly with distilled water. One side of the dialysis unit was filled with 1 ml of protein solution and the desired concentration of the detergent /1 ml/ was placed into the other side of the dialysis unit. The amount of the detergent bound to protein was calculated by determining the concentration of the detergent in the outside protein solution before and after dialysis. At detergent concentration below the CMC, 48 hours was enough to attain equilibrium. The casein concentrations were determined by the method of Lowry et al. [8] using bovine serum albumin as standard. The concentration of alkylpyridinium bromides was measured spectrophotometrically at 257 nm, which is a characteristic band for pyridinium ring, using calibration curves prepared for each detergent.

The concentration of DeG was measured by the antron method [9] using a calibration curve within a concentration range between 10^{-5} mole per liter and 2×10^{-5} mole per liter.

Critical micelle concentration /CMC/. The CMC's of alkylpyridinium bromides and DeG were determined by the surface tension dropweight method using a stalagmometr at 20°C.

The CMC values in a phosphate buffer, pH 6.8, ionic strength 0.1, of OPB, DePB, DPB, TPB and DeG were 5×10^{-2}, 2.8×10^{-2}, 1.0×10^{-2}, 9.0×10^{-4} and 2.1×10^{-3} mole per liter respectively.

Measurements of surface tension in casein solutions with cationic and non-ionic detergents. Surface tension measurements were carried out using two methods: the drop weight method and Wilhelmy slide method. Surface tensions of pure casein solutions were determined as well as of mixtures of casein with decyl- β -D-glucoside /DeG/, dodecylpyridinium bromide /DPB/ and tetradecylpyridinium bromide /TPB/. Calculated values of surface tensions in [dyne/cm] of all solutions are presented in table I.

1.2. Results

Table I

γ [dyne/cm]

casein	c = 0.2 %			1 %	
	53.30			49.98	
decyl- β -D- -glucoside DeG	c=0.016%	0.032%	0.048%	0.12%	0.16%
	42.15	33.47	28.35	26.68	26.68
casein - DeG	1 % : 0.016 %				
	42.39				
dodecylpyridinium bromide DPB	c= 0.015%	0.02 %		0.05 %	0.1 %
	70.81	69.99		65.83	59.43
casein - DPB	0.2% : 0.016%			0.2% : 0.02%	
	45.16			44.32	
tetradecylpyridinium bromide TPB	c= 0.01%	0.02%		0.05%	0.1%
	68.95	62.62		50.86	39.20
casein - TPB	0.2% : 0.01%			0.2% : 0.02%	
	46.08			44.85	

1.3. Discussion

1.3.1. Interaction of casein with detergents studied by equilibrium dialysis method.

The gel filtration of casein gave two protein peaks /fig.1/.

The protein designated as casein I was eluted at the void volume of the column and probably results from the agregation of protein. Rechromatography of casein II on analytical Sephadex G-150 column /Fig.2/ also gave two peaks. The molecular weight of casein eluted in peak II obtained by the method of Squire was 75000 /Fig.2/.

Binding isotherm. The binding of TPB and DPB in the phosphate buffer, pH 6.8, ionic strength 0.1, at room temperature, to native casein as a function of equilibrium detergent concentration is shown in Figures 3 and 4 respectively. The slope of the binding isotherms for TPB changes at point A /Fig.3/ at which the number of detergent moles bound per mol of protein, $\bar{\nu}$, is about 30.

Deviation from linearity at higher $\bar{\nu}$ values suggests a second set of binding sites on protein molecules. There are plateau regions both for TPB and DPB, designated as B and C respectively /Fig.3 and 4/. The binding in the plateau regions /B in Fig.3 and C in Fig.4/ of TPB and DPB can suggest conformational changes in the protein molecule.

The bindings of DePB and OPB at room temperature and pH 6.8, ionic strength 0.1 as a function of the detergent concentration in equilibrium are shown in Figures 5 and 6. The difference between DePB and OPB is not remarkable and the slopes of the isotherms slightly change at detergent concentrations approximately equal to their CMC.

The longer the hydrocarbon chain of alkylpyridinium bromides the lower the concentration of free detergent in equilibrium required to obtain the same level of binding.

The binding isotherms for decylglucoside at 10°C are shown in Fig.7. The average number of detergent molecules bound, $\bar{\nu}$, increases with the increase of detergent concentration in equilibrium. As can be seen in Fig.7 binding of DeG occours at a DeG concentration very close to its CMC value.

The correlation between binding and hydrocarbon chain length of alkylpyridinium bromides suggests that binding energy is derived from hydrophobic interaction.

On the other hand the result of non-ionic detergent binding such as decylglucoside to casein indicates that this detergent is bound with higher affinity than decylpyridinium bromide.

Fig.1. Sephadex G-150 gel filtration of casein. The column was equilibrated with phosphate buffer, pH 6.8, ionic strength 0.1 containing 0.01% NaN_3, and eluated with the same buffer.

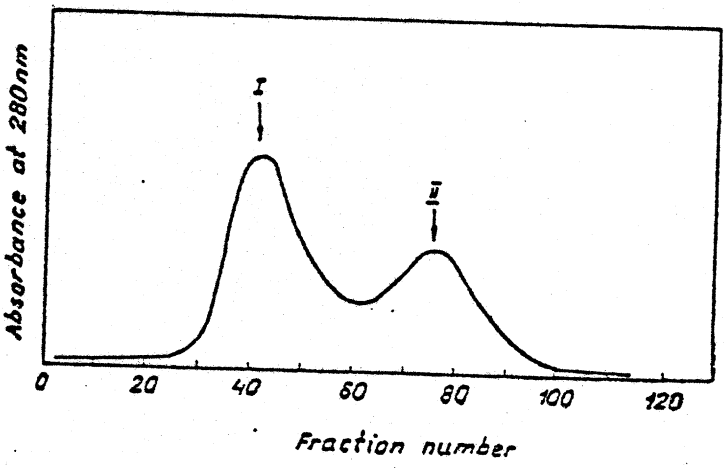
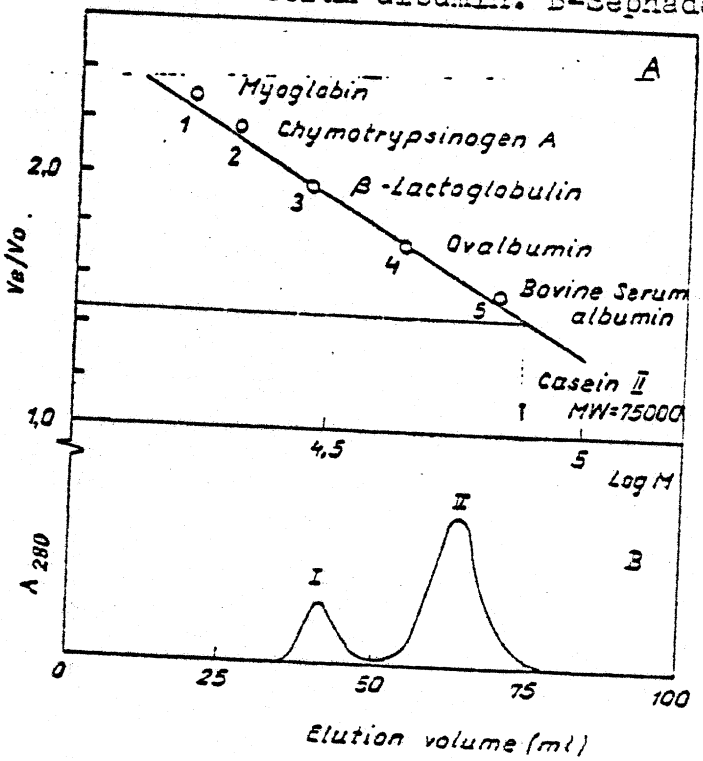


Fig.2. Molecular weight determination of casein II using Sephadex G-150 gel filtration. A-plots of the coefficient V_e/V_o versus log of molecular weight are given. The elution was performed with phosphate buffer, pH 6.8, ionic strength 0.1, marker proteins:1-myoglobin, 2-chymotrypsinogen, 3- β -lactoglobulin, 4-ovalbumin, 5-bovine serum albumin. B-Sephadex G-150 gel filtration of casein II.



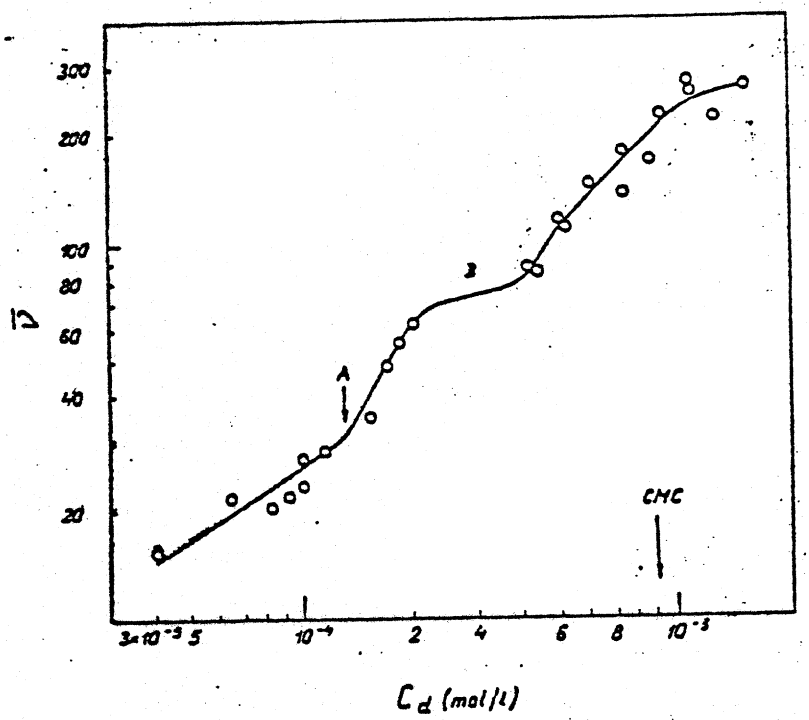


Fig.3. Logarithmic plot of the binding isotherm of casein at room temperature and pH 6.8 with tetradecylpyridinium bromide

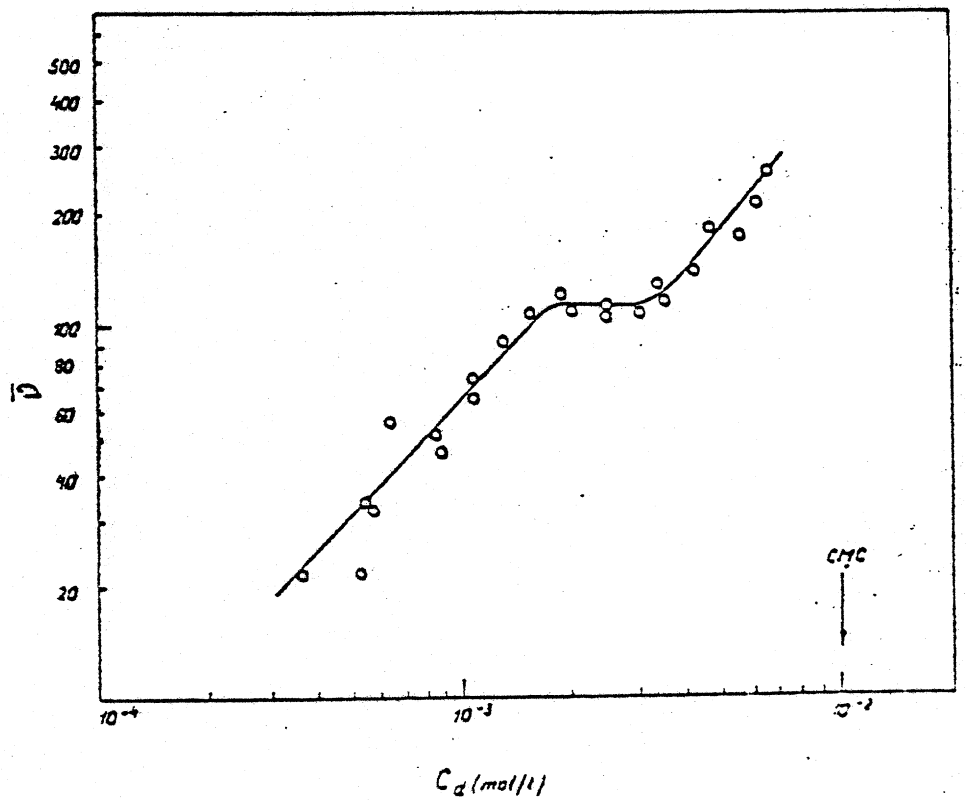


Fig.4. Logarithmic plot of the binding isotherm of casein at room temperature and pH 6.8 with dodecylpyridinium bromide

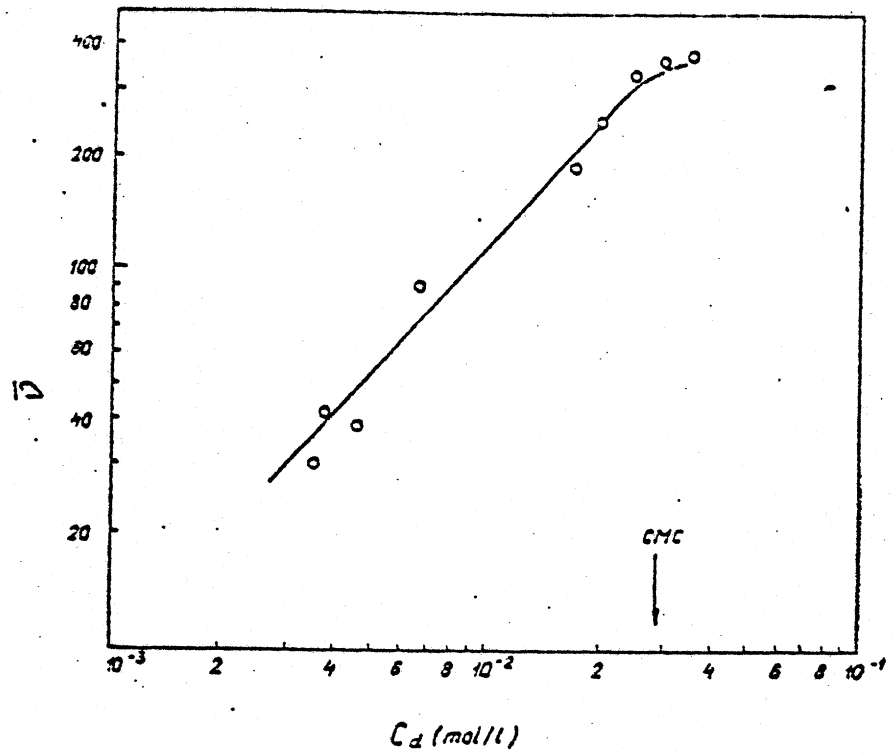


Fig.5. Logarithmic plot of the binding isotherm of casein at room temperature and pH 6.8 with decylpyridinium bromide

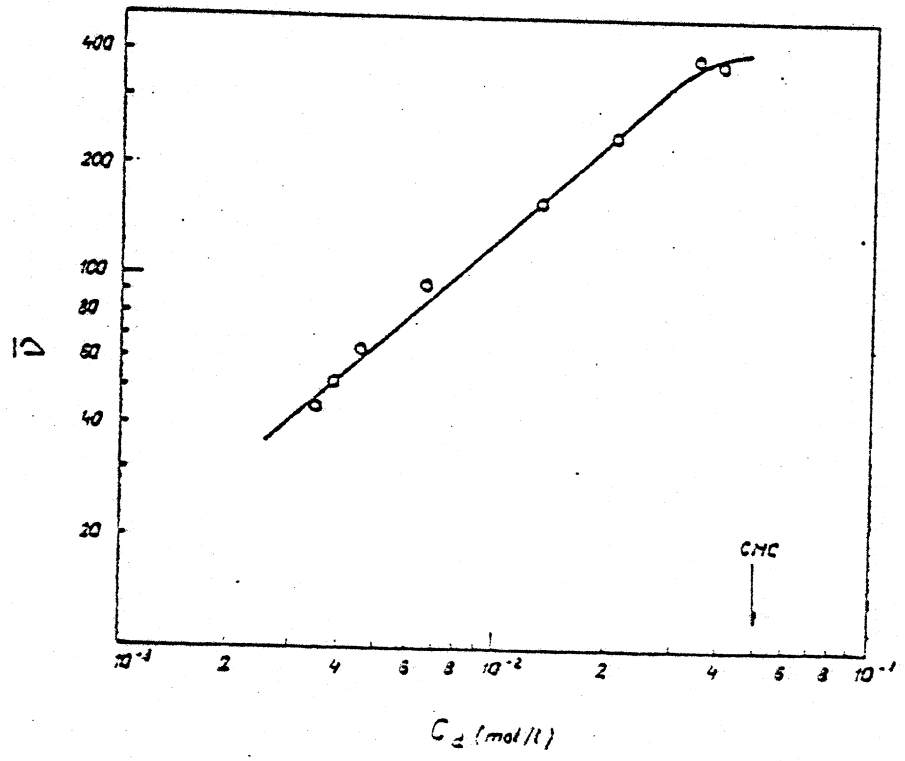


Fig.6. Logarithmic plot of the binding isotherm of casein at room temperature and pH 6.8 with octylpyridinium bromide

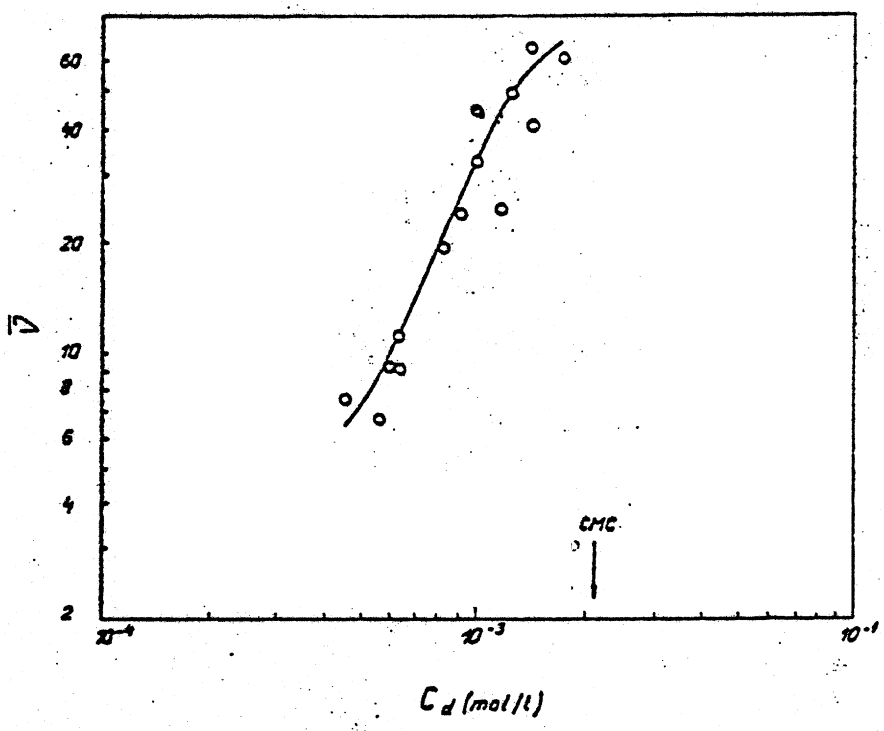


Fig.7. Logarithmic plot of the binding isotherm of casein at 10°C and pH 6.8 with decylglucoside.

The average number of cationic detergent molecules, such as alkylpyridinium bromides, bound to casein is on the same order as the number of anionic detergent molecules, such as alkylsulphates, bound to bovine serum albumin. However, this process occours for the cationic detergent and casein at 10-fold higher concentration than is required for alkylsulphates with corresponding hydrocarbon chain length. On the other hand the binding of DeG to casein is comperable to the binding of sodium decyl sulphate to bovine serum albumin [5].

1.3.2. Interaction of casein with cationic and non-ionic detergents studied by surface tension method.

As can be seen from Table I the addition of small amounts of non-ionic and cationic detergents causes an increase in surface activity of solution of about 7 - 9 dyne/cm. This witnesses the formation of specific casein-detergent interactions of hydrophobic character. The hydrophobic part of detergent squeezes itself into the casein micelle causing simultaneously its partial dehydration and also a decrease in its hydrophobic properties.

The casein-detergent complex created in this way is pushed

from the depths of the solution to the surface causing a decrease in surface tension of solution. The results of surfac tension measurements confirm the conclusions drawn from studie of casein-detergent interactions using the equilibrium dialysis method.

2. Interaction of casein with anionic detergents studied by surface tension and ultrasonic methods

2.1. Methods

The measurements of surface tensions of the solutions of casein with alkyl sulphates and with sodium derivatives of carboxylic acids with alkyl chains containing 7, 9 and 11 carbon atoms were carried out by the use of the drop weight method. The measurements were performed in a wide concentration range, for concentrations higher and lower than the CMC of these detergents. The measurements in the concentration range higher than CMC were therefore possible to perform because on the contrary to the cationic detergents, alkyl sulphates and carboxylates do not cause the precipitation of casein at a high detergent concentration. The measurements of ultrasound velocity in the solutions of casein with alkyl sulphates and carboxylates were performed with the apparatus described previously /part I/ at 25 ± 0.01°C using the same solutions which were used for the surface tension measurements.

2.2. Materials

The following alkyl sulphates were used for the measurements: sodium dodecyl sulphate /SDS/, sodium decyl sulphate /SDeS/ and sodium octyl sulphate /SOS/ which were made by Eastman Kodak Co. Sodium carboxylates such as: sodium laurate /LS/, sodium caprinate /CnS/ and sodium caprylate /ClS/ were synthesized from the corresponding carboxylic acids made by International Enzymes Limited. These acids were neutralized with stoichiometric amounts of NaOH of high purity. Casein was prepared for the measurements analogically as in point 1.

2.3. Results

The results of the measurements are presented in Figs.8 - 11

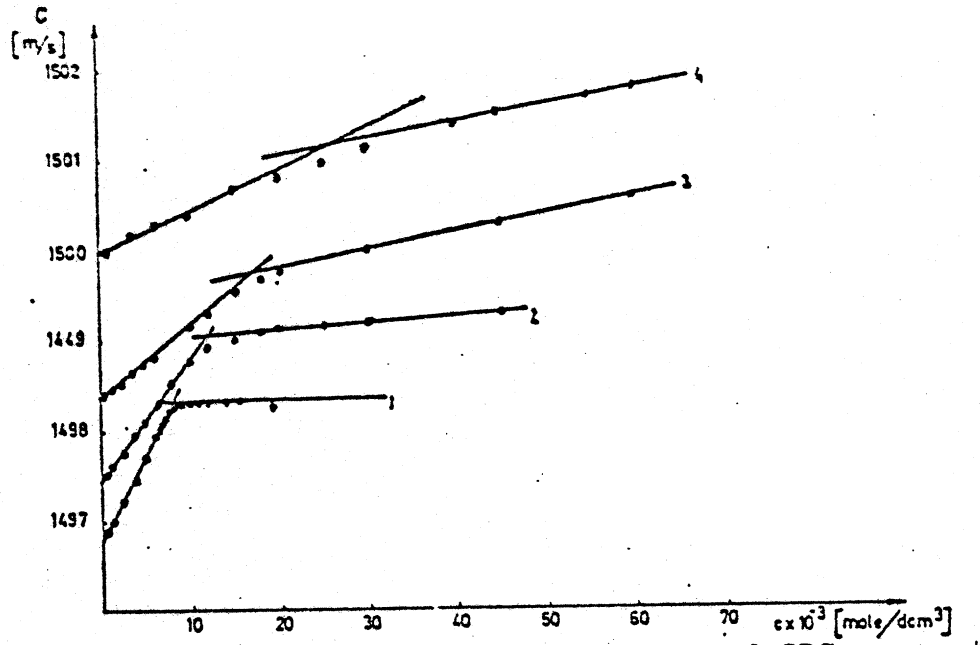


Fig.8. Ultrasound velocity as a function of SDS concentration for aqueous solutions of casein
1/ without casein
2/ 0.2% of casein
3/ 0.5% of casein
4/ 1% of casein

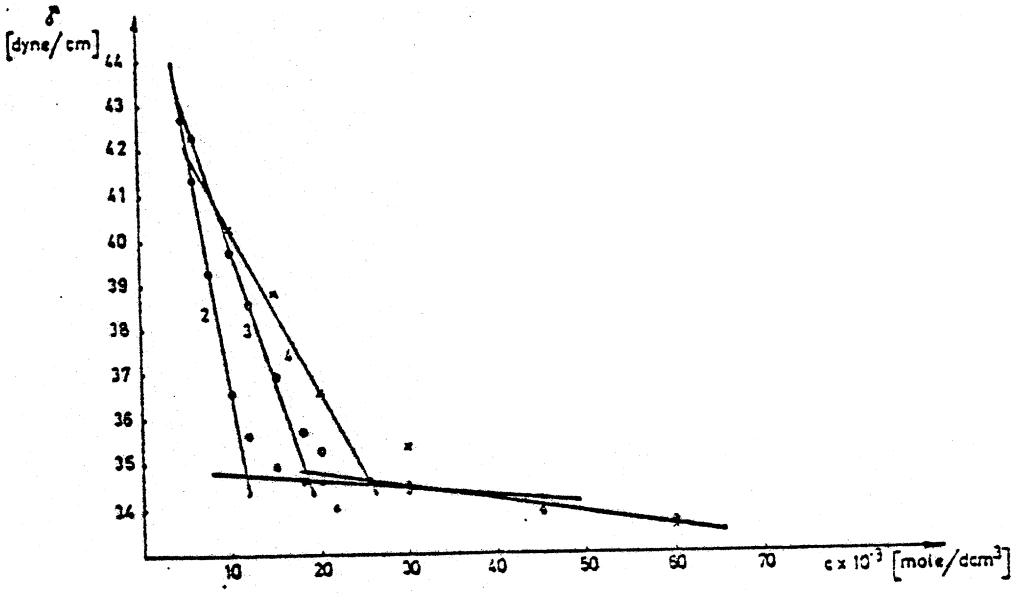


Fig.9. Surface tension versus SDS concentration for aqueous solutions of casein
2/ 0.2% of casein
3/ 0.5% of casein
4/ 1% of casein

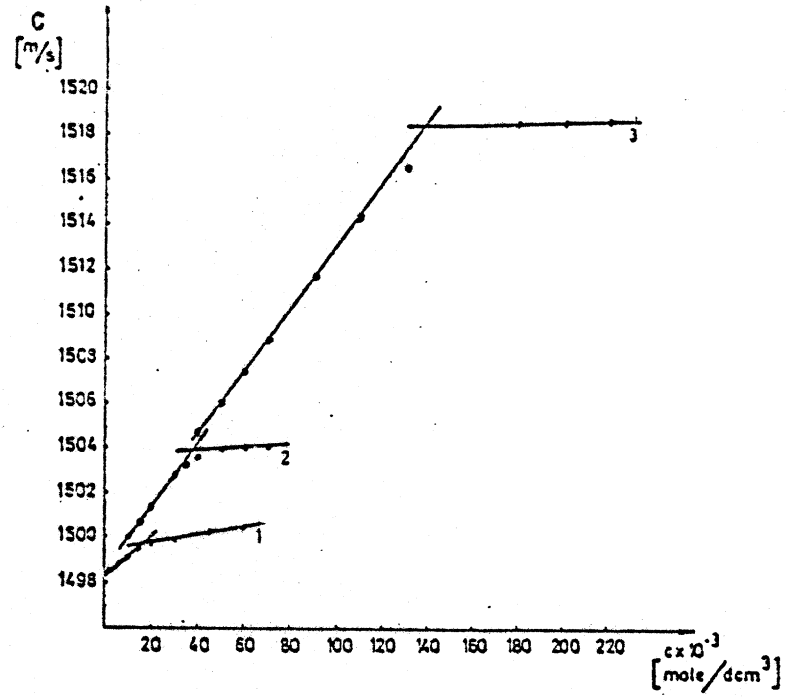


Fig.10. Ultrasound velocity as a function of concentration and kind of detergent added
1 - SDS
2 - SDeS
3 - SOS

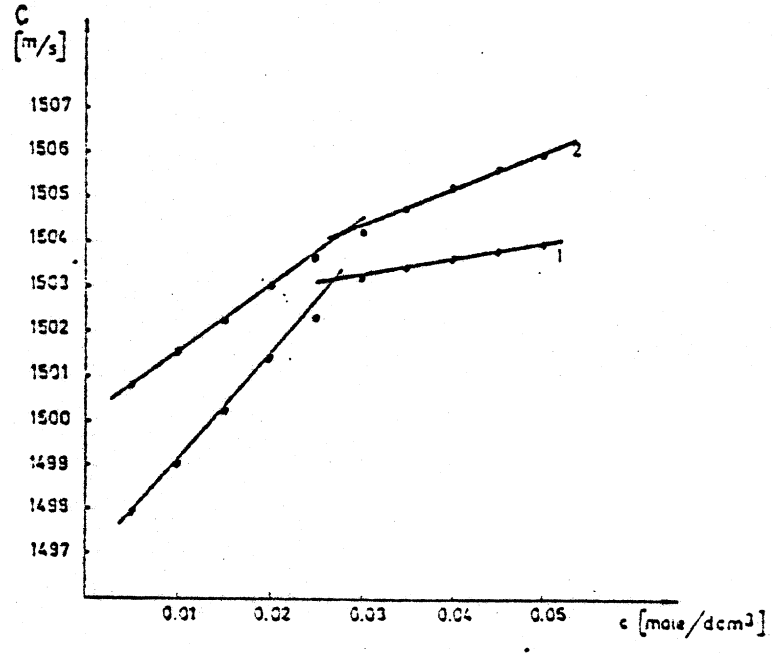


Fig.11. Ultrasound velocity versus concentration of sodium laurate
1/ without casein
2/ 1% of casein

The curves of the surface tension as a function of the detergent concentration for the solutions containing macromolecules have two characteristic points [4,5]. The first point is considered to be the detergent concentration at which the adsorption process /binding process/ begins and the second point is considered to be that at which the binding of maximum amounts of the given detergent molecules by the macromolecule occurs. For the concentrations higher than at the second characteristic point the surface tensions are the same as those of micelle solutions of the same detergent. It can be supposed therefore, that after complete saturation of the macromolecules with detergent the further addition of a surface active substance will cause its micellization. The second characteristic point on the surface tension curves can therefore be considered to be the critical micelle concentration of a given detergent in the presence of active macromolecules. On the ultrasound velocity curves the CMC is determined by the point of intersection of two straight lines to which the experimental results obtained for concentrations considerably higher and lower than CMC can be extrapolated [11,12]. As can be seen in Figs.8-11, the addition of casein to the solutions of all anionic detergents studied causes the shift of the CMC in the direction of higher detergent concentrations. Effects of a similar type were observed for systems of several polymers with alkyl sulphates [4,5]. The effect of broadening of ultrasound velocity and surface tension curves with the increase of the amount of protein in the solutions can be clearly seen. Accepting for a sure thing the fact that the CMC shift is connected with the formation of a complex from the macromolecule and part of the detergent molecules, then from the difference between the CMC of sole detergents and of the detergent solutions containing casein, the amounts of the particular detergents bound with a certain amount of protein can be determined. In Tables II and III the critical concentrations of micellarization for particular solutions with casein and without casein are presented as well as the differences of the CMC resulting from the presence of casein in the solution. Some numerical values of the CMC obtained from the surface tension and ultrasound velocity measurements

differ insignificantly and the CMC values for the detergent solutions without protein obtained from ultrasound measurements are in better agreement with the literature data [10-12] than are the analogous data obtained by means of the tensiometric method. The ultrasonic method should be used especially in the case of the detergent solutions containing the greater amounts of casein /~ 1%/ because surface tension curves are so strongly broadened that it is practically impossible to determine the CMC point, whereas on the ultrasound velocity curves this point can be determined quite exactly. Therefore, the results obtained from ultrasonic measurements were used for the determination of the CMC resulting from the presence of casein in the solution.

Table II.

	CMC [m/l]		
sodium laurate SL	% of casein	0 %	1 %
	sound velocity	$2.70 \cdot 10^{-2}$	$2.88 \cdot 10^{-2}$
	surface tension	$2.22 \cdot 10^{-2}$	$2.40 \cdot 10^{-2}$
	shift CMC		$1.8 \cdot 10^{-3}$
sodium caprinate SCn	% of casein	0 %	1 %
	sound velocity	$11.2 \cdot 10^{-2}$	$12.2 \cdot 10^{-2}$
	surface tension	$8.8 \cdot 10^{-2}$	$9.8 \cdot 10^{-2}$
	shift CMC		$1.0 \cdot 10^{-2}$
sodium caprylate SC1	% of casein	0 %	1 %
	sound velocity	$39.5 \cdot 10^{-2}$	$41.0 \cdot 10^{-2}$
	shift CMC		$1.5 \cdot 10^{-2}$

	CMC [m/l]			
	% of casein	0 %	0.2%	0.5%	1%
sodium dodecyl sulphate SDS	sound velocity	$8 \cdot 10^{-3}$	$12 \cdot 10^{-3}$	$17 \cdot 10^{-3}$	$26 \cdot 10^{-3}$
	surface tension	-	$12 \cdot 10^{-3}$	$18 \cdot 10^{-3}$	$25.5 \cdot 10^{-3}$
	shift of CMC		$4 \cdot 10^{-3}$	$1 \cdot 10^{-2}$	$1.8 \cdot 10^{-2}$
	% of casein	0 %	0.5%	1%	
sodium decyl sulphate SDeS	sound velocity	$3.3 \cdot 10^{-2}$	$3.7 \cdot 10^{-2}$	$4.5 \cdot 10^{-2}$	
	surface tension		$3.8 \cdot 10^{-2}$	$4.45 \cdot 10^{-2}$	
	shift of CMC		$5 \cdot 10^{-3}$	$1.2 \cdot 10^{-2}$	
	% of casein	0 %	0.5%	1%	
sodium octyl sulphate SOS	sound velocity	$13.6 \cdot 10^{-2}$	$13.8 \cdot 10^{-2}$		
	shift of CMC		$\sim 2 \cdot 10^{-3}$	$\sim 4 \cdot 10^{-3}$	

As can be seen in Tables II and III the sizes of the CMC shifts are dependent on the alkyl chain length of detergent and the influence of the chain is different depending on the kind of ionogenic group which is bound to this chain. In the case of alkyl sulphates the interaction between detergent and casein becomes stronger with the increase of the alkyl chain length; the number of detergent molecules bound with casein also increases. A similar effect was observed by Arai et al. [5] for the solutions of alkyl sulphates with polyvinylpyrrolidine. An opposite influence of the alkyl chain on the strenth of the interaction between detergent and casein was observed for the sodium derivatives of monocarboxylic acids. The number of carboxylate ions bound with casein decreases with the increase in the alkyl chain length. The differences observed in the interactions of casein with these two types of compounds can be explained by the stronger dehydration influence of $-SO_4$ group than of -COO- group on the casein micelle. Dehydration

of the casein micelle will occur mainly at its hydrophobic part where the weak interactions between water molecules and alkyl chains of protein /hydrophobic hydration/ occur. Water can be removed from the surface as well as from the interior of the micelle. In consequence the hydrocarbon chains of detergent have the possibility of penetrating the micelle interior and forming specific hydrophobic interactions with the hydrocarbon parts of casein. If the alkyl chain of detergent is longer the hydrophobic interactions are stronger and the number of detergent molecules interacting with casein becomes greater. The influence of the ionogenic group of the carboxylates on hydration of the surface as well as of the interior of the casein micelle is far weaker. Consequently the alkyl chains of the detergent will have a more difficult approach to the micelle interior because simultaneously there will exist repulsive forces caused by the molecules of the water which has remained in the interior of the micelle. This effect will increase with an increase of the chain length of the alkyl carboxylate. Accepting that the molecular weight of casein equals 75000 /see page 33 /, the numbers of moles of alkyl sulphates bound with one mole of casein are of the same order as the numbers of the molecules of these same detergents bound with albumin [1].

Conclusion

Casein interacts with all kinds of detergents: anionic, cationic and non-ionic. The specific interactions of detergents with casein depend on the penetration of the alkyl chains of detergents in to the interior of the casein micelle with simultaneous dehydration of the interior. Similar interactions can be expected in the case of lipid-casein systems occurring in milk and its products.

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Part IV. Investigations of the souring process of not fat milk

The souring process of milk was studied by the use of ultrasound velocity and pH measurements at 25°C. The measurements were carried out in fresh milk one hour after milking and in fresh milk after being kept for 24 hours in a thermostat at 5°C. The measurements were also performed on bottled milk which is supplied to the consumers usually 24 hours after milking and is pasteurized technologically. The results are presented in Fig.1.

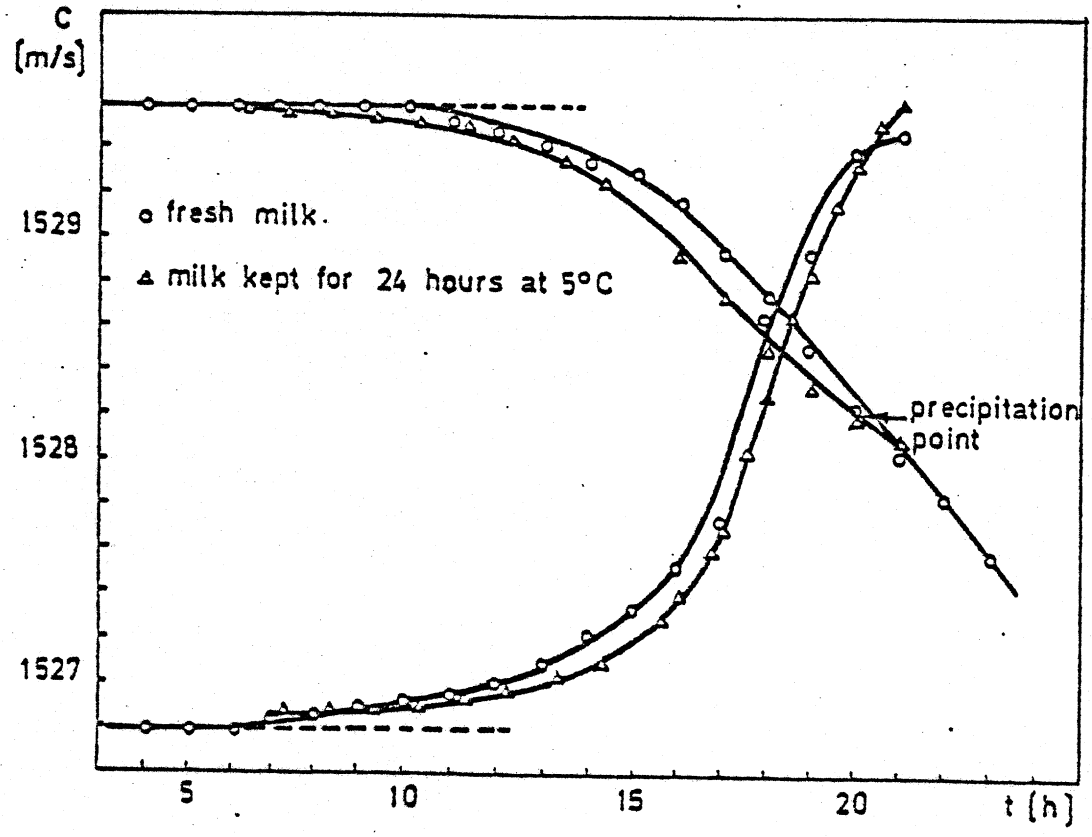

Fig1. Ultrasound velocity and pH versus storage time of milk.

As can be seen in Fig.1 the ultrasound velocity is constant during the first 6 hours after milking then increases insignificantly during the next 10 hours and after 16 hours of thermostating at 25°C until the moment of souring /20 hours/ the increase of ultrasound velocity is quite considerable and equals about 0.5 m/s during 1 hour. The values of ultrasound velocity measured for fresh milk and for milk thermostated

previously for 24 hours at 5°C differ little. A similar course of ultrasound velocity vs. storage time at 25°C coinciding almost in the entire range with the lower curve was obtained for bottled milk which was previously pasteurized. The comparison of the courses of ultrasound velocity and pH showes that the increase of ultrasound velocity is proportional to the decrease of pH in milk, so the increase of velocity is connected with the increase of the concentration of the sour components of milk which form mainly during the fermentation processes of lactose. With the aim of corraborating whether the above conclusion is true the measurements of ultrasound velocity and pH in fresh not fat milk to which acetic or lactic acid was added were performed. The results are presented in Fig.2.

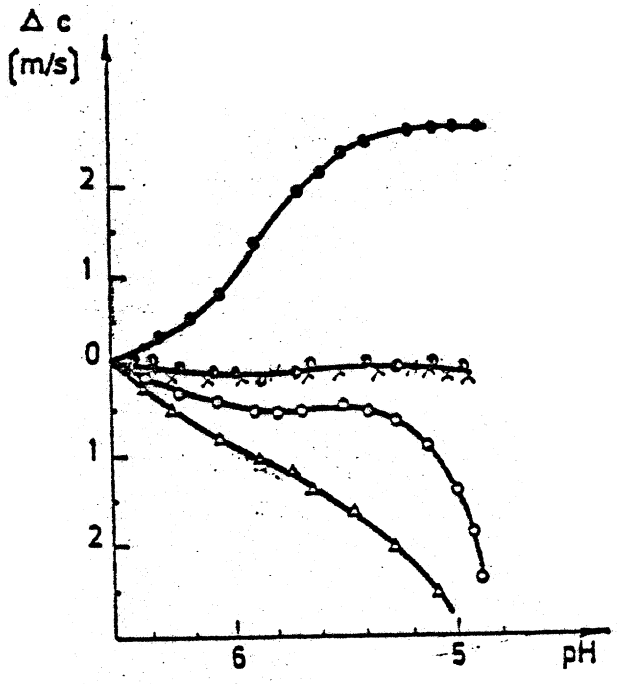

Fig.2. Changes of ultrasound velocity Δ v versus changes of pH occuring in milk:
● -during the storage process of milk
×, o -as a result of the addition of 0.36 n lactic acid
o -as a result of the addition of 0.4 n acetic acid
Δ - as a result of the addition of 0.2 n acetic acid

As can be seen in Fig.2 ultrasound velocity increases with the decrease of pH in milk only during the storage process. However, the addition of lactic acid causes of course a

decrease in the pH of milk but in spite of these pH changes from 6.6 until the precipitation point the ultrasound velocity almost does not change. In the case of the addition of acetic acid a rather marked decrease in velocity is observed. This decrease is higher for acetic acid of lower concentration. This is connected with the greater dilution of milk by the titrating agent. The results of measurements indicate that the increase of the concentration of acids forming in milk as s result of fermentation processes has not a great influence on the observed ultrasound velocity changes during the storage process of milk. It seems true that the main factors which cause the rapid increase of ultrasound velocity of milk in a given pH range are the processes of rearientation and increase in the order of protein macromolecules. The shape of the curves of ultrasound velocity vs. storage time in the pH range 6 - 5 is similar to that which was obtained for surface active substances near the CMC [1,2]. It can be supposed that the physicochemical processes which occur near the precipitation point of protein are similar to the processes which are observed in the solutions of surface-active substances in a concentration range near the CMC.

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Professor Dr. B.Zapiór
Principal Investigator

Professor Dr. Z.Wojtaszek
Director of the Institute